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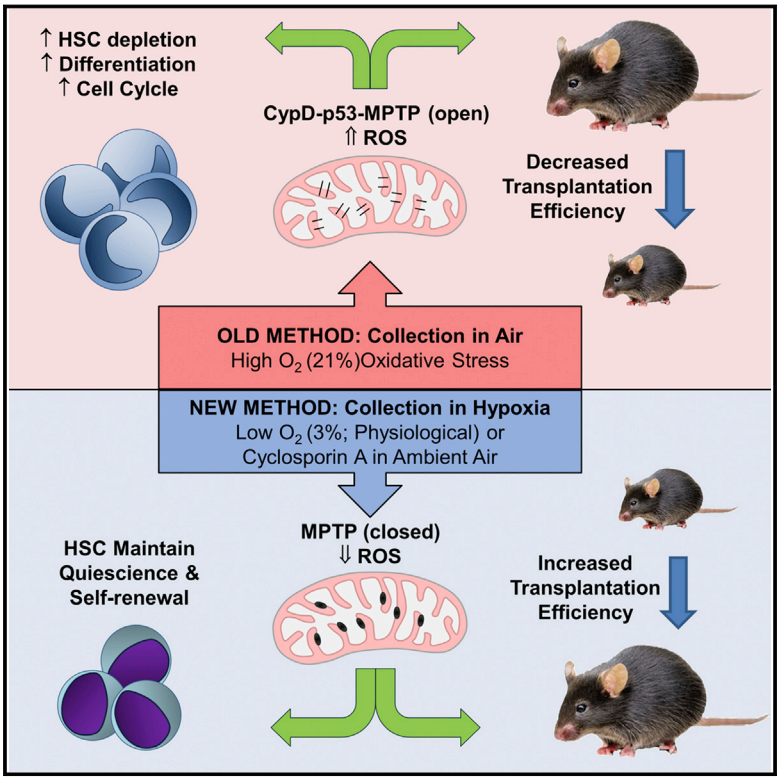
Enhancing Hematopoietic Stem Cell Transplantation Efficacy by Mitigating Oxygen Shock

Graphical Abstract



Highlights

- Exposure to ambient air compromises HSC recovery from bone marrow and cord blood
- HSC numbers are grossly underestimated because of collection in air
- The decrease is mediated by ROS linked to the *CypD-p53*-MPTP axis, *miR210*, and *hif-1α*
- HSC transplantation efficiency is enhanced with cells collected in cyclosporin A

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In Brief

Ambient air compromises the survival of bone marrow and cord blood hematopoietic stem cells through the activation of the mitochondrial permeability transition pore. Inhibition of this process with cyclosporin A protects the cells, promoting transplantation efficacy.

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Enhancing Hematopoietic Stem Cell Transplantation Efficacy by Mitigating Oxygen Shock

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SUMMARY

Hematopoietic stem cells (HSCs) reside in hypoxic niches within bone marrow and cord blood. Yet, essentially all HSC studies have been performed with cells isolated and processed in non-physiologic ambient air. By collecting and manipulating bone marrow and cord blood in native conditions of hypoxia, we demonstrate that brief exposure to ambient oxygen decreases recovery of long-term repopulating HSCs and increases progenitor cells, a phenomenon we term extraphysiologic oxygen shock/stress (EPHOSS). Thus, true numbers of HSCs in the bone marrow and cord blood are routinely underestimated. We linked ROS production and induction of the mitochondrial permeability transition pore (MPTP) via cyclophilin D and p53 as mechanisms of EPHOSS. The MPTP inhibitor cyclosporin A protects mouse bone marrow and human cord blood HSCs from EPHOSS during collection in air, resulting in increased recovery of transplantable HSCs. Mitigating EPHOSS during cell collection and processing by pharmacological means may be clinically advantageous for transplantation.

INTRODUCTION

Hematopoietic stem cells (HSCs) give rise to all the blood-forming elements, and their presence in bone marrow (BM), mobilized peripheral blood (PB), and cord blood (CB) has allowed their harvesting for the treatment of malignant and non-malignant disorders. However, the rarity of HSCs, particularly in CB grafts, can be a limitation of hematopoietic cell transplantation (Ballen et al., 2013). Uncovering mechanisms in HSC biology can identify new strategies to enhance the number and function of HSCs and improve engraftment efficacy. While HSCs and hematopoietic progenitor cells (HPCs) proliferate better in vitro in hypoxia than in normoxia (Bradley et al., 1978; Broxmeyer et al., 1985; Danet et al., 2003; Lu and Broxmeyer, 1985; Smith and Broxmeyer, 1986), all HSC/HPC studies are performed after cell collection and processing in ambient air (~21% O_2) regardless of subsequent processing in hypoxia or air. The BM and CB environment where HSCs reside is extremely hypoxic compared to air (Morrison and Scadden, 2014; Nombela-Arrieta et al., 2013; Sjöstedt et al., 1960; Spencer et al., 2014). Thus, HSC collection in air is grossly hyperoxic compared to the BM microenvironment.

Stem cells rely heavily on glycolysis instead of mitochondrial respiration for bioenergetic demands (Xu et al., 2013). Mouse long-term repopulating HSCs (LT-HSCs) harbor significant numbers of mitochondria that appear to be inactive or "nascent" and poised for rapid activation (Mantel et al., 2010). This is associated with initial differentiation of quiescent LT-HSCs into "activated" HSCs and short-term repopulating HSCs (ST-HSCs). In mice, this is linked to lack of CD34 expression and increased CD150 expression (Anjos-Afonso et al., 2013; Doulatov, et. al., 2012; Ema et al., 2006; Mantel et al., 2010) and is also thought to involve reactive oxygen species (ROS) (Jang and Sharkis, 2007; Lewandowski et al., 2010), a normal byproduct of respiration that promotes HSC differentiation (Broxmeyer and Mantel, 2012; Ito et al., 2004, 2006; Tothova and Gilliland, 2009; Yalcin et al., 2008). We recently linked mitochondrial respiratory dysfunction and ROS overproduction to depletion of LT-HSCs, effects partially rescued by the ROS scavenger N-acetylcysteine (Mantel et al., 2012). Therefore, we hypothesized that suppressing ROS during HSC collection and processing in a more physiological low O_2 environment (hypoxia) might offer protection from mitochondrial dysfunction and result in increased HSC recovery.

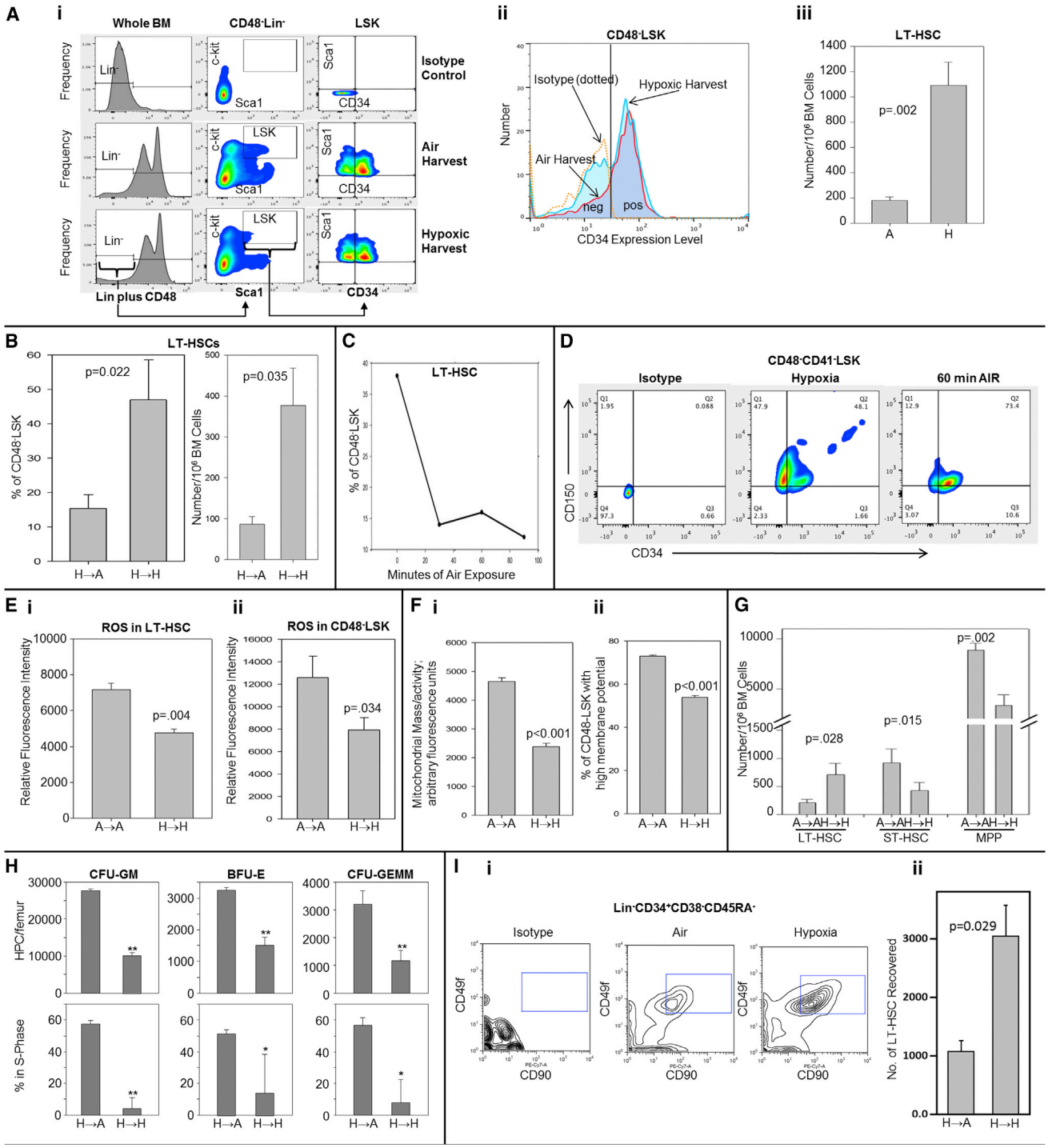

Figure 1. BM Harvest and Processing in Hypoxia or Ambient Air

(A) BM was harvested and processed in a hypoxic chamber (3% O_2, 5% CO_2, N_2 balance) or ambient air (~21% O_2). (i) Flow cytometric density dot plots are representative of six independent experiments. LT-HSCs are defined as $CD34^-CD48^-$LSK. (ii) Relative frequency histograms. Bar indicates CD34-positive or negative staining based on isotype control antibody. (iii) Number of LT-HSCs collected, per 10^6 BM cells, when harvested and processed in air (A) or in the hypoxic chamber (H); mean ± SE for six independent experiments (statistics determined by Mann-Whitney method).

(B) LT-HSCs collected from BM harvested in an hypoxic chamber and divided into two; one was removed from the chamber and immediately exposed to air for 60 min (H→A), and the other was left in the chamber for 60 min (H→H) before further staining and processing. Chart bars are mean ± SE for three independent experiments.

(legend continued on next page)

Here, we provide a rigorous analysis of how brief exposure of HSCs to air affects the efficiency of HSC collection and transplantation success, and we describe the molecular mechanisms underlying it. We show that exposure to air during collection limits the yield of HSCs from BM and CB, and we name this phenomenon "extraphysiologic oxygen shock/stress" (EPHOSS). EPHOSS effects are mediated by ROS production linked to cyclophilin D (CypD), p53, and the mitochondrial permeability transition pore (MPTP). Importantly, inhibition of EPHOSS using cyclosporin A (CSA) enhances the yield of HSCs and the efficacy of their transplantation. This phenomenon, suggesting that greater numbers of HSCs reside in hematopoietic tissues and that their in vivo metabolism is different from the one ex vivo in air, raises questions regarding the in vivo relevance of studies of HSCs and HPCs collected in air. Moreover, hematopoietic cell transplantation, especially where donor HSCs are limited, may be improved if EPHOSS is prevented or attenuated by collection and processing of cells under hypoxia or, alternatively, in air in the presence of CSA or through other pharmacological targeting of the MPTP.

RESULTS

Effects of "Hypoxic Harvest"

To limit ROS production and HSC differentiation, mouse BM was collected and processed under constant hypoxia (3% O_2) and compared to air-harvested BM; either one femur was harvested inside a hypoxic chamber and the other in air, or BM was collected in the chamber and aliquots exposed to ambient air or left in the chamber for processing. Figure S1A shows the hypoxic chamber used for these studies. Most importantly, all reagents and supplies were equilibrated to hypoxia (3% O_2) for at least 18 hr prior to use.

Up to 5-fold greater numbers of phenotypically defined mouse BM LT-HSCs ($Lin^-Sca1^+c\text{-}kit^+$ [LSK] $CD48^-CD34^-$) were recovered by harvesting and maintaining cells in constant hypoxia (3% O_2) compared to air (Figure 1A). Similar increases were noted when cells were collected and processed in hypoxia (H→H), compared to cells collected in hypoxia then placed in air (H→A) for 60 min prior to assessment (Figure 1B), an effect rapidly lost if cells were exposed to air for as short as 30 min (Figure 1C). This pattern was also observed if CD150 (Oguro et al., 2013) was used to phenotypically delineate LT-HSCs instead of CD34 ($CD150^+CD48^-CD41^-$LSK; Figure S1B). As the $CD48^-CD41^-$LSK population displays less CD34 on their surface, they increase CD150 expression (Figure 1D). This verifies that phenotypically defined mouse BM LT-HSCs are recovered in greater numbers when harvested in hypoxia and prevented from any exposure to air, regardless of phenotypic markers utilized. ROS levels were increased in LT-HSCs and $CD48^-$ LSK cells (containing HSCs and HPCs) harvested in air compared to hypoxia (Figure 1E). Elevated mitochondrial activities (Figure 1Fi) and increased numbers of primitive cells with hyperpolarized mitochondria (Figure 1Fii) were found in air-harvested BM. Thus, decreased LT-HSCs correlated with augmented mitochondrial activity in air-exposed BM cells. Numbers of ST-HSCs ($CD34^+CD48^-$LSK cells) and multi-potent progenitors (MPPs; $CD48^-Lin^-c\text{-}kit^+Sca1^-$) were increased after air exposure (Figures 1G and S1C), suggesting rapid differentiation of LT-HSCs to ST-HSCs and HPCs in air. Hypoxic-harvested BM contained reduced numbers and cell cycling of immature subsets of multi-cytokine-stimulated HPCs (Figure 1H; assessed by colony assays), consistent with reduced ROS-mediated cytokine-induced signaling and differentiation (Sattler et al., 1999). Thus, true numbers and frequency of LT-HSCs, as they exist in their native low [O_2] environment, have previously been greatly underestimated and numbers and cycling status of HPCs overestimated in mouse BM. Collection/processing of mouse BM at 5% O_2 did not increase numbers of HSCs as did 3% O_2 (Figure S1D).

Human CB is also hypoxic compared to ambient air (Sjöstedt et al., 1960). Thus, human CB was collected with syringes designed to greatly minimize exposure of CB to air (Experimental Procedures) and was typically transferred within 10 min into the hypoxic chamber for further processing. Human-CB-derived HSCs, identified as $Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ (Notta et al., 2011; Doulatov et al., 2012), resulted in ~3-fold greater recovery compared to cells handled in air (Figure 1I), consistent with mouse BM hypoxic harvests, suggesting exquisite sensitivity of HSCs to EPHOSS and demonstrating that EPHOSS is not restricted to BM.

(C) Length of air exposure on numbers of LT-HSCs, which were collected after harvest in a hypoxic chamber (zero time) and aliquots exposed to air for 30, 60, or 90 min before staining and processing (one experiment). Logistically, it was difficult to do a time point much less than 30 min.

(D) Flow cytometric density dot plots of CD34 and CD150 expression in $CD48^-CD41^-$LSK cells. With this antibody combination, LT-HSCs are defined as $CD150^+CD34^-CD48^-CD41^-$LSK (Oguro et al., 2013). Density plots represent two similar experiments.

(E) (i) ROS levels in LT-HSCs harvested and processed in ambient air (A→A) or in hypoxia (H→H) (three independent harvests on the same day; mean ± SD). (ii) ROS levels in $CD48^-$LSK; mean ± SD for three independent experiments (one mouse per collection per experiment on different days).

(F) (i) Mitochondrial activities, measured by MitoTracker Green FM mean fluorescent intensity in $CD48^-$LSK BM cells collected and processed in ambient air (A→A) or hypoxia (H→H); mean ± SD for three independent experiments. (ii) Percentage of $CD48^-$LSK cells with hyperpolarized mitochondrial membrane potential using JC-1 probe in BM collected and processed in ambient air or hypoxia; mean ± SD for three independent experiments.

(G) Numbers of LT-HSCs, short-term HSCs (ST-HSCs; $CD34^+CD41^-CD48^-$LSK), or multi-potent progenitors (MPPs; $CD48^-Lin^-Sca1^-c\text{-}kit^+$); mean ± SD for six independent experiments (one mouse per experiment each harvested, processed, and analyzed on different days).

(H) Absolute numbers per femur (upper graphs) and cycling status (% in S phase as determined by high-specific-activity tritiated thymidine kill assay; lower graphs) of HPCs for BM cells collected and processed in hypoxia (H→H) or collected under hypoxia and placed in air for 60 min. (H→A) and then cultured in hypoxia (5% O_2). Mean ± SE for three mice each from one experiment. *$p < 0.05$; **$p < 0.001$. Results were reproduced in four additional experiments with three mice/group each.

(I) (i) Representative flow cytometric contour plots of human LT-HSCs ($Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ as per Notta et al., 2011) from CB collected under hypoxic conditions with half left in hypoxia for processing (H→H) and half left in air for 60 min. (H→A). (ii) Number of phenotyped human CB-derived HSCs per 10^6 total cells; mean ± SD for four independent CB harvests.

See also Figure S1.

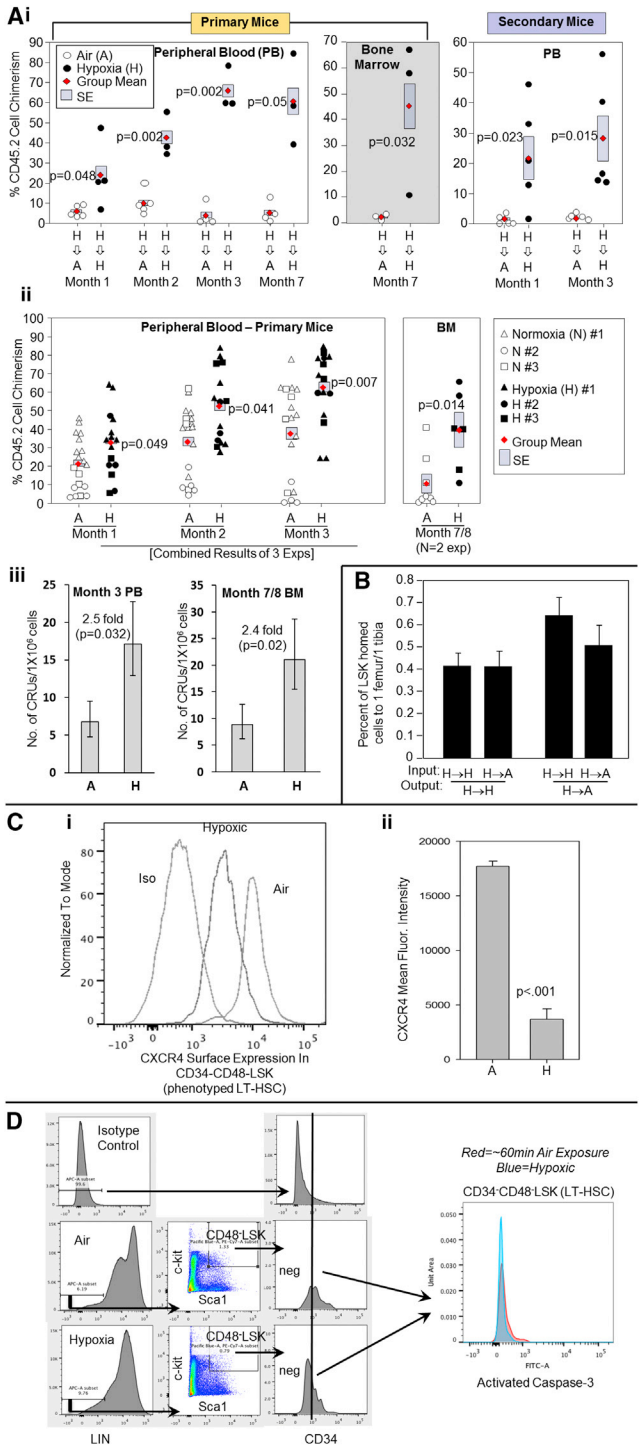

Figure 2. Competitive Mouse HSC Repopulation, HSC Homing, Apoptosis, and CXCR4 Expression

(A) Competitive HSC engraftment. Donor cells ($CD45.2^+$) and competitor cells ($CD45.1^+$; competitor cells were collected in air and injected either in hypoxia or in air immediately after donor cells were injected) were infused into 950-cGy irradiated dual $CD45.2^+/CD45.1^+$ F1 recipients (150,000 donor and 150,000 competitor cells). (i) BM cells were harvested in hypoxia and cells split so that half were processed and injected in hypoxia and half subjected to air for 60 min prior to processing and injecting in air. Mean ± SE of $CD45.2^+$ (donor cell) chimerism (one experiment) for numbers of mice evaluated. Open circles, hypoxia (H)→air (A); closed circles, (H→H). (ii) Combined results of two or three separate engrafting studies as noted in the text. (iii) CRUs calculated from LDA as per Antonchuk et al. (2002) (n = 3–4 mice per group at each cell concentration infused for each of two experiments) for month 3 peripheral blood and month 7 or 8 for BM. p value is based on Poisson statistics.

(B) Homing of cells collected in hypoxia and then left to be processed and injected under hypoxia (H→H for input) or collected under hypoxia and then exposed to room air for 60 min before processing and injecting cells in air (H→A for input). Cells were analyzed 24 hr after injection by removing two femurs plus two tibias from ten mice under hypoxia and splitting the cells into two, one half which was left at hypoxia for staining and assessment (H→H for output) and one half placed into air for 60 min for staining and assessment (H→A for output). Lin^- BM cells were injected, and % LSK cells homed (output to input LSK) showed no significant differences in any of the groups by ANOVA analysis.

(C) (i) Representative relative frequency histograms of CXCR4 surface staining intensity in LT-HSCs. (ii) Mean fluorescence staining intensity of four BM harvests (mean ± SD).

(D) Flow cytometric assessment of apoptosis using antibody to activated caspase-3. Data represent two similar experiments.

See also Figure S2.

Since phenotype does not always recapitulate function, especially under stress (Broxmeyer et al., 2012), we assessed HSC function by competitive transplantation. Mouse BM was harvested, processed, and injected into mice in a hypoxic chamber, or cells were collected in hypoxia and exposed to air for >60 min before processing and transplantation in air. A custom mouse respiration device facilitated tail vein injection inside the hypoxic chamber (Figure S1E). Recipient mice were only briefly (<10 s) exposed to low O_2. Competitor BM was collected in air and infused after the donor cells. Engraftment was significantly increased when donor BM was harvested, processed, and transplanted in low O_2, compared to BM harvested in hypoxia and then exposed to air (Figure 2Ai), consistent with increased numbers of functional HSCs being present in hypoxic-harvested BM. Low-level engraftment was seen with air-exposed donor cells, while engraftment of hypoxia-harvested, processed, and intravenously injected donor BM cells resulted in quite significant enhancement in PB and BM chimerism in primary mice, as well as enhanced repopulation in secondary mice. Two other similar engraftment experiments were performed, including one with "air-only" harvest compared to "hypoxic-only" harvest. Chimerism of air-harvested donor cells was higher in the two latter experiments than that shown in Figure 2Ai, even though similar donor-to-competitor numbers were used. Combined results of all three experiments demonstrated significant enhancement for hypoxia-harvested, processed, and injected BM donor cells (Figures 2Aii and S2A; competitive repopulation units [RUs], as calculated by the method of Harrison and Astle, 1997). Limiting dilution analysis (LDA) at month 3 for PB and month 7 or 8 for BM performed for two of the experiments shown in Figure 2Aii for hypoxia- versus air-harvested cells demonstrated increased competitive repopulating units (CRUs) of 2.5- and 2.4-fold, respectively (Figures 2Aiii and S2B). There was no significant difference in lymphoid and myeloid end-stage cell graft contribution in air- versus hypoxia-harvested, processed, and injected cells in the engrafted mice (Figure S2C). Enhanced engraftment

of hypoxic-harvested/processed cells was not due to homing (Figure 2B; "homed" LSK cells were collected in hypoxia and then analyzed either in hypoxia or 1 hr after placement in air, with no differences noted for "homed" cells either way). Enhanced engraftment did not correlate with enhanced CXCR4 expression, which was decreased on hypoxic- compared to air-harvested LT-HSCs (Figure 2C). Apoptosis, assessed by intracellular active caspase-3 levels, was not different when hypoxic-harvested BM was exposed to air for 60 min (Figures 2D, S2D, and S2E). So, cell death could not explain loss of HSCs when BM was exposed to air. The picture emerging from our studies is that harvesting donor BM in air, or even brief exposure to air (i.e., in response to EPHOSS), has a rapid, deleterious effect on numbers and repopulating potential of HSCs.

Mechanisms of EPHOSS were assessed for biologic insights, and as an alternative means of collecting cells to mimic effects seen in low O_2 for practical applicability. Physiological damage from heart attack, stroke, and other ischemic events occurs upon restoration of circulation and tissue "re-oxygenation." So-called ischemia-reperfusion damage is believed to be initiated by a burst of oxygen radicals rapidly produced by mitochondria (Kalogeris et al., 2012; Perrelli et al., 2011), with some similarity to air BM harvest. Induction of the MPTP is implicated in mechanisms of ischemia-reperfusion damage (Griffiths and Halestrap, 1995; Kim et al., 2003; Lim et al., 2010). This may be similar to what HSCs experience upon harvest in ambient air, with induction of the MPTP via oxidative stress. While oxidative stress favors MPTP induction resulting in mitochondrial swelling and OXPHOS uncoupling (Halestrap and Davidson, 1990) leading to apoptosis and necrosis (Vaseva et al., 2012), MPTP opening can also be intermittent/transient and function in a regulatory capacity conducive to regulation of differentiation of stem cells. We hypothesized that the MPTP is involved in EPHOSS because of similarities to ischemia-reperfusion.

MPTP Is Key to the EPHOSS Mechanism

A key regulatory component of the MPTP is peptidyl-prolyl *cis-trans* isomerase, or cyclophilin D (CypD; also called cyclophilin F), which is encoded by the *Ppif* gene and regulates MPTP induction (Tanveer et al., 1996). Oxidative stress facilitates recruitment of mitochondrial CypD to the inner membrane and promotes MPTP induction (Connern and Halestrap, 1994). CSA, a small-molecule inhibitor of CypD, binds CypD, antagonizes MPTP induction (Halestrap and Davidson, 1990; McGuinness et al., 1990; Nicolli et al., 1996), and prevents ischemia-re-oxygenation damage (Hausenloy et al., 2012). US Food and Drug Administration-approved CSA is being tested for treatment of heart attack and stroke and is an immunosuppressant in graft versus host disease (GVHD) for HCT (Junghanss et al., 2012; Kikuchi et al., 2012). To test MPTP involvement in EPHOSS mechanisms, and for possible protective effects of MPTP inhibition, BM was harvested in air with CSA. Harvest of cells with CSA resulted in ~4-fold significant increase in recovery of LT-HSC numbers (Figures 3A–3C). LT-HSCs declined rapidly when BM was harvested in air without CSA (Figure 3Ciii), consistent with kinetics in hypoxic/air-harvest experiments (Figure 1). CSA harvest also suppressed multi-cytokine-induced proliferation of granulocyte-macrophage CFUs (CFU-GM) (Figure 3D), implicating CypD and the MPTP in cytokine signaling/function. Delaying addition of CSA for 15 min while collecting mouse BM in air did not rescue the EPHOSS effect and resulted in decreased numbers of LT-HSCs and increased numbers of ST-HSCs and MPPs, along with increased ROS in the three cell types (Figure S3).

Non-MPTP-related immunosuppressive CSA effects are mediated by inhibition of the calcineurin pathway (Liu et al., 1991). To determine if this pathway is involved in CSA protection against EPHOSS, BM was harvested in air with a calcineurin inhibitor, FK506, which does not inhibit the MPTP and is used to address specificity of agents thought to affect the MPTP (Friberg et al., 1998). "FK506 harvest" did not protect LT-HSCs from EPHOSS (Figure 3E), confirming CSA protects LT-HSCs from EPHOSS via suppression of the MPTP. BM was also harvested in air in the presence of carboxyatractylate (CAT), an agent that binds to adenine nucleotide translocase, stabilizes it in the *c*-conformation, and favors MPTP opening (Halestrap and Brenner, 2003). "CAT harvest" resulted in loss of LT-HSC recovery (Figure 3F), which was greater than that by air harvest alone. ROS levels were reduced in LT-HSCs and HPCs when BM was harvested with CSA, but they increased in the presence of CAT (Figure 3G), further supporting MPTP-opening-mediated ROS generation in molecular mechanisms of EPHOSS. To determine if "CSA harvest" protects functional HSCs from EPHOSS, we performed competitive repopulation experiments. BM harvested in the presence of CSA from donor mice that were pretreated with CSA had enhanced competitive engraftment as determined by donor cell chimerism (Figure 3Hi), CRUs from LDA (Figures 3Hii and S4A), RUs calculated by the method of Harrison and Astle (1997) (Figure S4B), and enhanced secondary repopulation (Figure 3Hi). Lymphoid and myeloid cell numbers of CSA versus non-CSA collected, processed, and injected cells in the engrafted mice were similar (Figure S4C).

To assess potential clinical applicability, we performed engraftment studies of CSA (diluted in DMSO) versus DMSO control collection and processing of human CB. In five separate collections, there were increased numbers of human $CD34^+$ cells and LT-HSCs and decreased MPP numbers (Figure 4A). There was enhanced engraftment in NSG mice of CSA collected and processed CB, as assessed by LDA for severe combined immunodeficiency (SCID) repopulating cells (SRCs) (Figures 4Bi–4Biii and S5Ai–S5Aiii), without significant differences in lymphoid and myeloid end-stage cell graft contribution (Figure S5B). Data in Figures 3 and 4A–4C demonstrate that regulation of the MPTP by CypD is an important mechanism of EPHOSS, and suppression of CypD opening of the MPTP with CSA may have practical clinical value.

The MPTP-CypD-p53 Axis Is Involved in EPHOSS

We reasoned that *CypD* gene deletion, which prevents MPTP induction (Baines et al., 2005; Baines, 2010; Kalogeris et al., 2012), might protect against EHPOSS. $CypD^{-/-}$ mice are protected against MPTP-dependent ischemia-reperfusion damage (Baines et al., 2005; Schinzel et al., 2005; Nakagawa et al., 2005). LT-HSC recovery was significantly increased (Figure 4Ci), and ROS levels in LT-HSCs significantly reduced (Figure 4Cii),

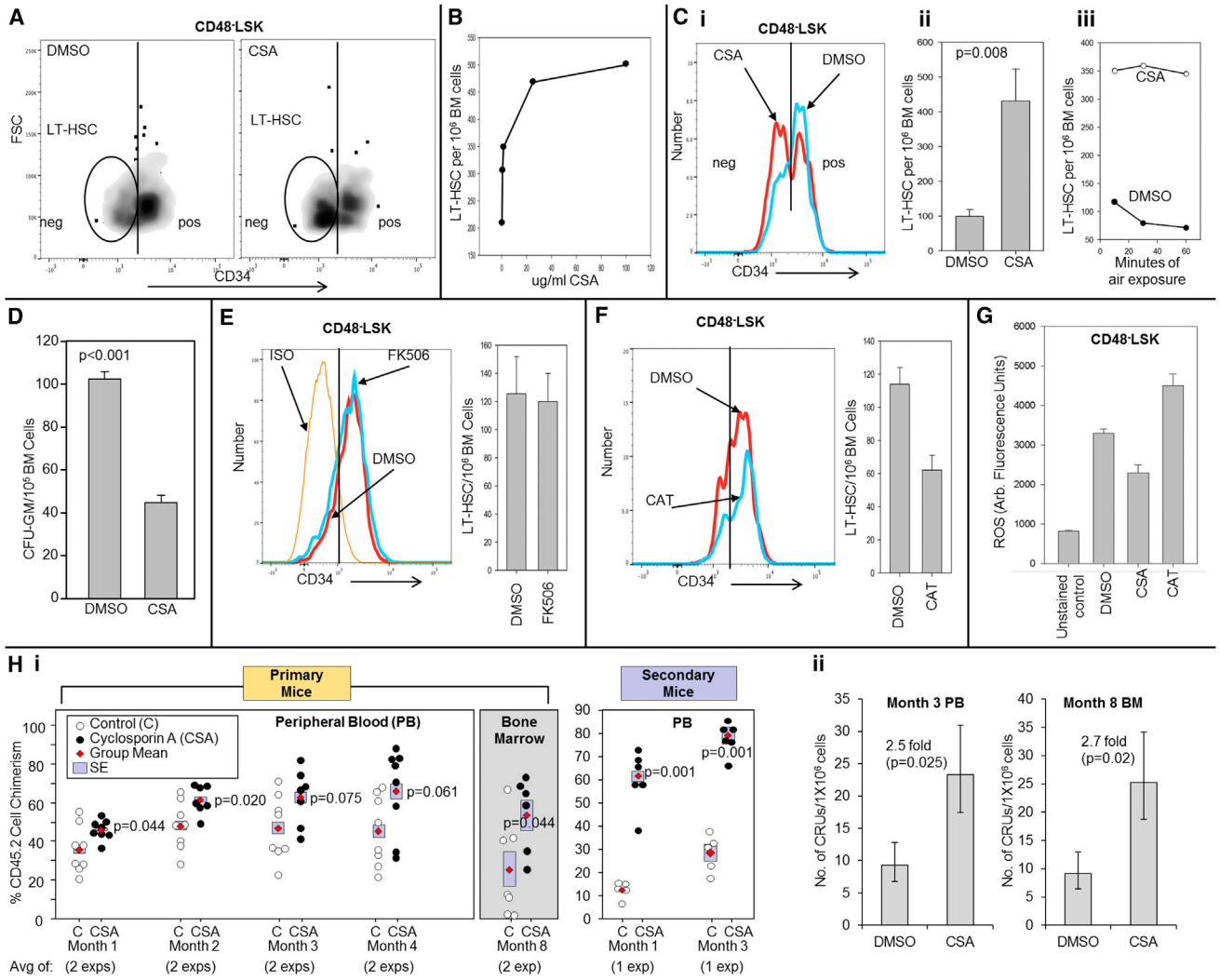

Figure 3. MPTP in EPHOSS: Effects of Cyclosporin A

(A) Effects of BM harvest in air in presence of 50 μg/ml cyclosporin A (CSA-harvest) or DMSO control on CD34 expression levels in CD48-LSK cells. For the CSA group, mice used for cell collection were injected with CSA (see Experimental Procedures). Mice in the control group were injected with control medium. Representative flow cytometric density dot plot for three independent harvests. LT-HSCs are noted in circles.

(B) Dose-response of CSA on LT-HSC collection (n = 1 experiment).

(C) Relative frequency histogram representative of three independent harvests in DMSO or 50 μg/ml CSA. (ii) Average LT-HSCs collected after DMSO or CSA-harvest (three independent experiments; mean ± SD). (iii) Effect of time of air exposure on BM harvested in the presence of either DMSO or CSA (one experiment).

(D) Effects of CSA or DMSO harvest on granulocyte-macrophage colony-stimulating factor (GM-CSF) plus stem cell factor (SCF)-induced CFU-GM colony formation (mean ± SD from six mice each in a total of two experiments).

(E and F) Effects of FK506 (E) or carboxyatractylate (CAT) (F) harvests shown as a relative frequency histogram from flow cytometric data. Data in (E) and (F) are representative of two independent experiments, each with similar results. Bar charts are quantitation (mean ± range) for two experiments.

(G) Effect of DMSO, CSA, or CAT harvest on ROS levels in $CD48^-$ LSK cells (mean of two independent experiments ± range).

(H) (i) Effect of CSA harvest, compared to DMSO (control; C) harvest, on HSC engraftment in competitive repopulation transplant assay (mean % $CD45.2^+$ donor cell chimerism ± SE for numbers of mice shown at each point). Results for months 1–4 and 8 for primary mice are for two experiments, while those at 1–3 months for secondary mice are for one experiment. (ii) CRUs calculated by LDA analysis respectively at 3 months for peripheral blood (PB) and at 8 months for BM (n = 3–5 mice per group at each cell concentration infused for each of two experiments, mean ± SE). p value is based on Poisson statistics.

See also Figures S3 and S4.

in *CypD*$^{-/-}$ BM harvested in air. HPC numbers were lower in *CypD*$^{-/-}$ BM (Figure 4D), and colony formation of CFU-GM in response to stimulation by a different multi-cytokine combination was also inhibited (Figure 4E), analogous to results of CSA treatments (Figure 3D). This strongly implicates CypD- and MPTP-mediated ROS production in cytokine signaling/stimulation of HPC proliferation. Chimerism (Figure 4Fi) and LDA to calculate CRUs, along with analysis of lymphoid/myeloid engraftment (Figures 4Fii and S5C–S5F), demonstrated increased engrafting capability of *CypD*$^{-/-}$ BM HSCs without

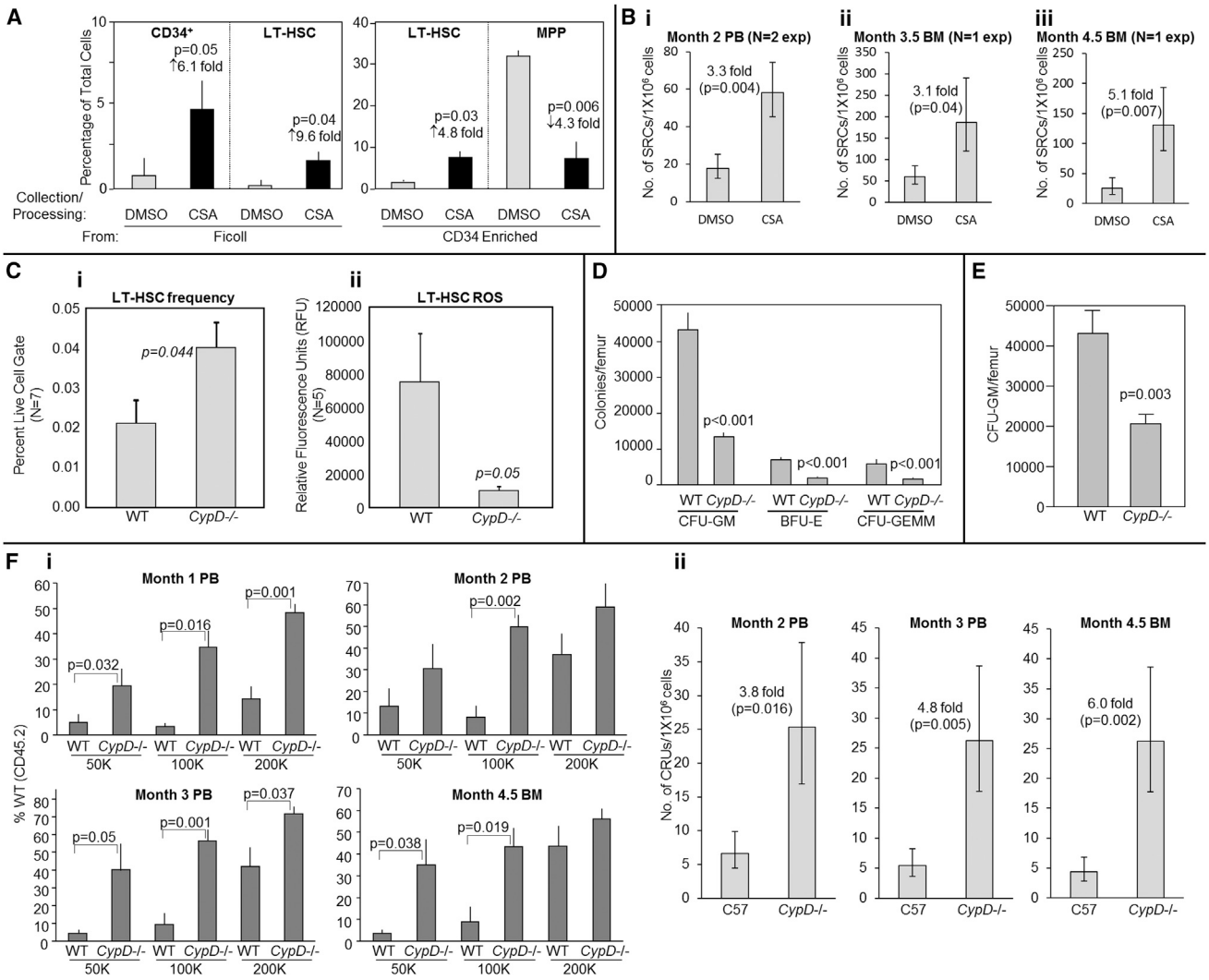

Figure 4. Effect of Human CB CSA and Mouse BM *cyclophilin D* (*CypD*)$^{-/-}$ Collections

(A) Effect of CSA collection on CB CD34$^+$ cells, LT-HSCs, and MPPs. Results are from five different CB collections (mean ± SE).

(B) CRUs calculated from LDA (n = 3–5 mice per group at each cell concentration for each of two separate CB collections (mean ± SE). (Bi) Combined results of two experiments at 2 months for PB (mean ± SE). (Bii) Results of one of the CB collections at 3.5 months for BM (mean ± SE). (Biii) Results of the other CB collection at 4.5 months for BM (mean ± SE) (Bii and Biii results for BM were not combined, as the percent chimerism for both was largely different, precluding averaging the results for LDA).

(C) (i) Effect of *CypD* gene deletion on phenotyped LT-HSC recovery when BM is harvested in air (n = 7 experiments, mean ± SE). (ii) ROS levels in LT-HSC cells harvested from five of the seven different experiments shown in (i) (mean ± SE).

(D) Recovery of multi-cytokine-stimulated CFU-GMs, erythroid burst-forming units (BFU-Es), and CFU-GEMMs (granulocyte, erythrocyte, monocyte, and megakaryocyte CFUs) (average of six WT and ten *CypD*$^{-/-}$ mice in a total of two experiments expressed as mean ± SE).

(E) GM-CSF plus SCF-induced CFU-GM colony formation (mean ± SE) in BM from the same animals as in (D).

(F) (i) Percent PB chimerism of *CypD*$^{-/-}$ BM cell engraftment at limiting dilution analysis for months 1–3 in PB and at 4.5 months in BM (four to five mice/group at each cell concentration for one experiment; mean ± SE). (ii) CRUs as calculated for months 2 and 3 PB and month 4.5 BM by LDA for *CypD*$^{-/-}$ BM cell engraftment (mean ± SE).

See also Figures S4 and S5.

changes in numbers of lymphoid and myeloid cells. The importance of the MPTP in EPHOSS is demonstrated in Figures 4C–4F.

ROS can be produced by mitochondrial and non-mitochondrial sources. Mitochondrial ROS production in cells predominantly occurs in the electron transport chain and is therefore closely linked to respiratory activity, which in turn depends on coupling to ATP synthesis. We wondered if *CypD*$^{-/-}$ cells may have any abnormalities in respiration and OXPHOS and if these abnormalities could clarify mechanisms behind lower ROS generation in *CypD*$^{-/-}$ cells. We used a Seahorse XF96 flux analyzer to assess cell respiration. Because LT-HSCs are rare, we used a

surrogate hematopoietic cell, a strategy used previously (Mantel et al., 2012). Non-mitochondrial respiration in $CypD^{-/-}$ cells was similar to that in wild-type (WT) cells and well coupled to ATP synthesis as determined by inhibition of respiration by rotenone and sensitivity to oligomycin A, respectively (Figure S6). Basal respiration and maximal respiratory capacity was higher in $CypD^{-/-}$ cells compared to WT cells, suggesting a potential mechanistic link between mitochondrial electron transport chain/respiration regulation and reduced ROS generation in hematopoietic cells in the absence of CypD.

p53 deletion is one of few gene deletions resulting in increases in HSC numbers and engraftment (Copley et al., 2012; Nii et al., 2012; Rossi et al., 2012). Recent findings indicate that p53 may facilitate MPTP opening (Vaseva et al., 2012; Zhen et al., 2014). We hypothesized that $p53^{-/-}$ might protect HSCs from EPHOSS by suppressing MPTP opening. We also considered if resistance to EPHOSS could have had a role in interpretation of results, leading to the idea that $p53^{-/-}$ BM contains increased HSCs. We now report that expected increases in HSC numbers in $p53^{-/-}$ mouse BM are not apparent with BM harvested and assayed in 3% O_2 and is only apparent when BM is harvested and exposed to air (Figure 5A), suggesting a role for p53 in HSC biology directly related to EPHOSS. This may influence current concepts about the effects of $p53^{-/-}$ on HSC biology in vivo. We also noted decreased numbers and cell cycle of $p53^{-/-}$ HPCs compared to WT if BM was harvested in low [O_2] and exposed to air (Figure 5B). However, if $p53^{-/-}$ BM was harvested and cultured in low [O_2], there were increased numbers and cycling of progenitors compared to WT BM harvested and cultured the same way. Therefore, analogous to effects of hypoxic harvest of LT-HSCs from $p53^{-/-}$ mice (Figure 5A), interpretation of effects of $p53^{-/-}$ on functional HPC is highly dependent on whether BM was exposed to air, and it suggests involvement of a p53-CypD-MPTP axis in EPHOSS mechanisms.

HIF-1a, miR210, and EPHOSS

Most tissues in the human body reside in an O_2 environment considerably lower than that of ambient air. If cells/tissues like HSCs that normally reside in low-O_2 conditions are collected and studied in ambient air, this could lead to incomplete understanding of their biology/biochemistry unless EPHOSS is considered.

To gain additional support for this idea, we investigated two other gene-deletion models connected to the biology of hypoxia: the hypoxamir miR210 and HIF-1α (Chan and Loscalzo, 2010; Devlin et al., 2011; Speth et al., 2014; Zhang et al., 2012). We wished to see if mir210 and HIF-1α may be linked to EPHOSS and to discern if we would detect differences in HSC and HPC numbers from these gene-knockout mice compared to control mice when cells are collected in air versus 3% O_2. Deletion of either gene suppressed hypoxic-harvest-enhanced recovery of LT-HSCs (Figures 5Ci and 5Cii) with amelioration of decreased recovery of ST-HSCs and MPPs by hypoxic harvest compared to WT controls. Hypoxic harvest of cells from control mice resulted, as already shown in Figure 1H, in decreased functional HPCs from WT BM, an effect not seen in either $miR210^{-/-}$ or $hif\text{-}1\alpha^{-/-}$ (Figures 5Di and 5Dii). These data implicate miR210 and hif-1α in EPHOSS, but they do not yet elucidate mechanistic links. The experimental results obtained were dependent on BM harvest conditions, similar to what was observed for the $CypD^{-/-}$ and $p53^{-/-}$ models.

DISCUSSION

Our studies illuminate several seminal concepts. First, HSCs from mouse BM or human CB can be collected in greater numbers than previously recognized and that have been until now significantly underestimated by routine harvesting and processing in air. This information could lead to improvements especially for CB-hematopoietic cell transplantation, where low cell numbers collected in single units create clinical limitations (Ballen et al., 2013). Moreover, there is a potential for rapid clinical translation because CSA, which protects HSCs from EPHOSS during air harvest via inhibition of the MPTP, is already used clinically as an immunosuppressant.

On a more fundamental level, our study also provides insight into the cellular effects of oxygen exposure, linking hypoxia, CypD, and the MPTP in cytokine signaling and function and suggesting new types of "mitochondria-centric" cytokine signal transduction pathways that precisely link proliferation and differentiation signals to cellular bioenergetics, metabolism, and programmed cell death via the MPTP. Moreover, using four gene-deletion mouse models, we highlight how interpretation of experimental results of gene-deletion models can be influenced by EPHOSS. While *CypD* or *p53* deletion (Figure S7) have an EPHOSS-protective effect, *miR210* and *hif-1*α deletion (Figure S7) abrogates protection afforded by hypoxic harvest. This reveals a specificity of EPHOSS requirements relative to several different factors that will have to be further evaluated.

Because different adult stem cells naturally exist in hypoxic niches, EPHOSS may be relevant to other stem cells routinely harvested in air. Embryonic stem cells in the inner cell mass of blastocysts, as well as cancer stem cells, all reside in hypoxic environments (Brown and Giaccia, 1998; Hill et al., 2009; Millman et al., 2009; Mohyeldin et al., 2010), and ROS is important in growth, differentiation, and regulation of these cells (Tothova and Gilliland, 2009). Human embryonic stem cells derived from eight-cell embryos thawed from liquid N_2 under 5% O_2 better maintained pluripotency, although in those studies, embryos were originally harvested in air (Lengner et al., 2010). Another important point to consider here is that there is much information about the metabolic regulation of HSCs (Suda et al., 2011), and some may now need to be rigorously re-evaluated in the context of EPHOSS. One example of issues arising from our findings is that metabolic profiling for development of "personal" therapeutic strategies to target cancer stem cells (Hsu and Sabatini, 2008; Kamleh et al., 2011; Wood et al., 2014) may not accurately represent the metabolism of these cells as they exist in their native hypoxic environments, because they are harvested and studied in air. It is also possible, and indeed highly likely, that EPHOSS will influence the metabolism and differentiation of other cell types, including lymphocytes, monocytes/macrophages, neutrophils, fibroblasts, and others, since these cells also reside in hypoxic environments.

Another point deserving exploration is the detrimental effect of aging on HSCs and other tissue-specific stem cells (Chambers

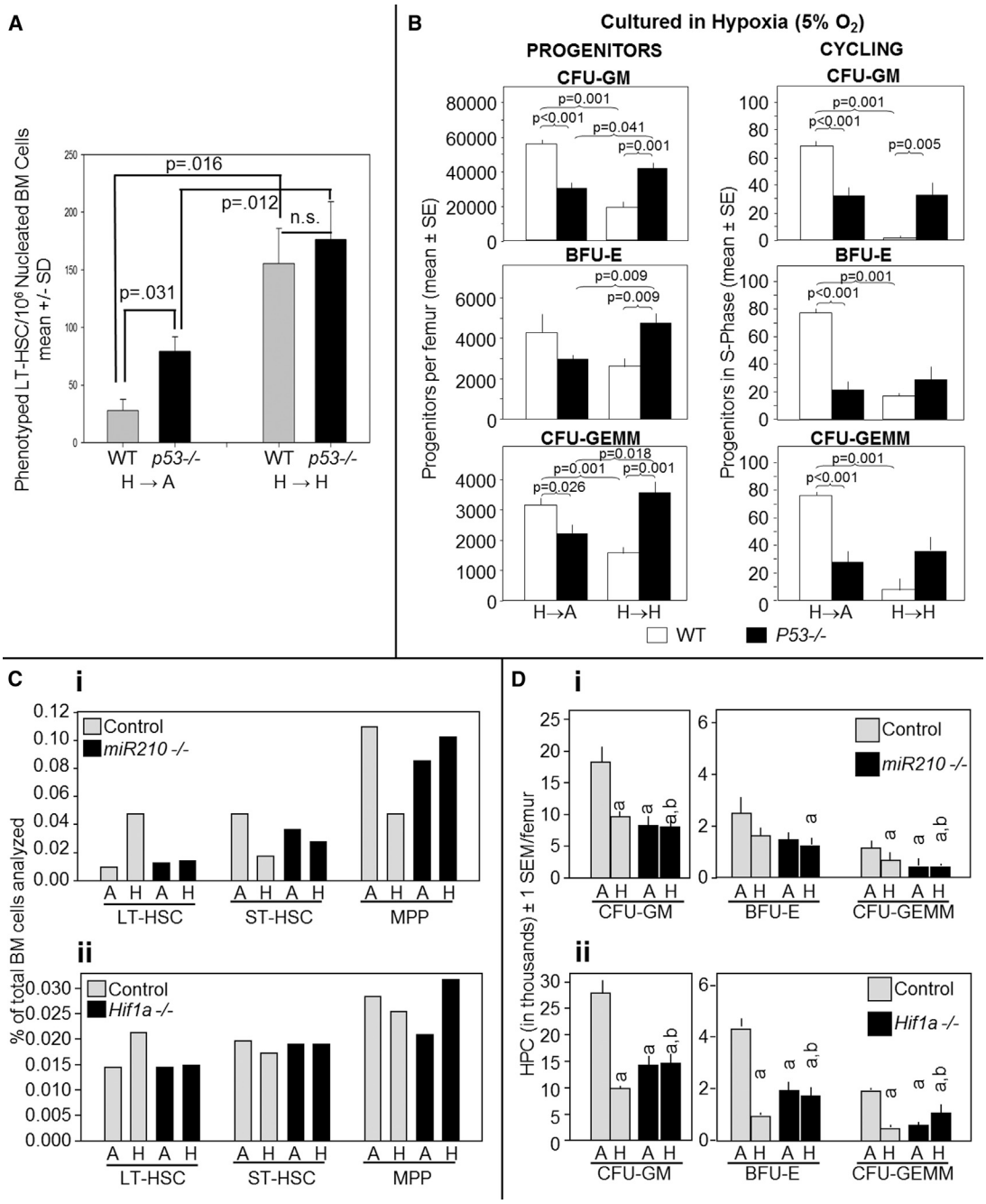

Figure 5. Effect of *p53*, *miR210*, or *hif-1α* Gene Deletion

(A) Effect of *p53* gene deletion on hypoxic (H→H) and air (H→A) BM harvest on phenotyped LT-HSC recovery for independent BM harvests from six $p53^{-/-}$ and three littermate control wild-type (WT) mice. BM from each mouse was harvested and maintained in the hypoxic chamber for 60 min before staining and fixation (H→H) or was exposed to air for 60 min before staining and fixation (H→A). Mean LT-HSCs per 10^6 nucleated BM cells ± SD for each group.

(B) Effect of *p53* gene deletion on multi-cytokine-stimulated progenitor cell recovery (left) or cell-cycle status (right; high-specific-activity tritiated thymidine kill assay). BM was from the same animals harvested in (A), and cells were cultured in hypoxia (5% O_2).

(C) Phenotyped LT-HSC, ST-HSC, and MPP recovery for $miR210^{-/-}$ (i), or $hif\text{-}1\alpha^{-/-}$ (ii) BM. Cells from one femur were collected and processed in air (A), and cells from the contralateral femur were collected and processed in hypoxia (H). One of two experiments with similar results for (i) and one experiment for (ii).

(D) HPC recovery from $miR210^{-/-}$ (i) or $hif\text{-}1\alpha^{-/-}$ (ii) BM harvested and processed in air (A) or hypoxia (H) and cultured in hypoxia (5% O_2). One of three reproducible experiments with three mice/group (mean ± SE) for each experiment for the $miR210^{-/-}$ mice in which one femur was collected in air and the contralateral femur collected in hypoxia, with one of the other two experiments done in a similar manner and one experiment done with collection in hypoxia and processing in air versus collection and processing in hypoxia. One of two reproducible experiments for $hif\text{-}1\alpha^{-/-}$ mice (n = 3–5, mean ± SE) with harvest in air versus hypoxia. a, significant ($p < 0.01$) compared to WT A control; b, not significant ($p > 0.05$) compared to $hif\text{-}1\alpha^{-/-}$ or $miR210^{-/-}$.

et al., 2007; Ergen and Goodell, 2010; Mantel and Broxmeyer, 2008; Mantel et al., 2011; Sudo et al., 2000). ROS is considered a major driver in aging (Finkel et al., 2007; Harper et al., 2004), and it is possible that stem cells from aged animals are more vulnerable to EPHOSS-linked ROS production when studied in air. Thus, cells collected from aged animals or humans using efforts that mitigate EPHOSS effects may have greater therapeutic potential.

In summary, we believe that knowledge of EPHOSS will have widespread ramifications for studies of cellular metabolism and function in many stem/progenitor and other cell systems.

EXPERIMENTAL PROCEDURES

Animals

Mice used for most BM harvests and for transplantation were female C57BL6/J and 6–8 weeks of age, while mice for the other experiments were males and females with ages ranging from 6 to 20 weeks of age. Mice were age and sex matched. In some cases, controls were littermates. Constitutive *CypD*$^{-/-}$ (*Ppif*$^{-/-}$) mice (B6; 129-Ppif$^{tm/JMOL}$/J) were purchased from The Jackson Laboratory. We verified knockout of CypD in hematopoietic cells by western blotting (Figure S7A). *p53*$^{-/-}$ mice on a Bl/6 background were as reported previously (Jacks et al., 1994; Vemula et al., 2012), and knockout was verified by PCR (Figure S7B). Tamoxifen-induced conditional *hif1α*$^{-/-}$ mice were as previously reported, where ERT-2 *Cre*$^{+}$*Flox*$^{+/-}$ and their ERT-2 *Cre-Flox*$^{+/+}$ littermate controls both received tamoxifen and knockout was confirmed by PCR (Speth et al., 2014). *miR210*$^{-/-}$ mice were developed in the laboratory of Nabeel Bardeesy (Massachusetts General Hospital, Boston, MA) and successful knockout determined by PCR (Figure S7C) (see Supplemental Experimental Procedures). Bl/6, B6BoyJ, F1, and NSG mice were from our animal cores.

Flow Cytometry

This was done using an LSRII cytometer or FACS Calibur (Becton Dickenson) and fluorophor-conjugated antibodies used for mouse BM cell phenotyping (Mantel et al., 2010, 2012) (see also Figure S1C). Antibodies used for human phenotyping (Notta et al., 2011) were anti-lineage cocktail, CD34, CD38, CD45RA, CD90, and CD49f (BD Biosciences). For mitochondrial mass, membrane potential, and ROS analysis (Mantel et al., 2010, 2012), we used MitoTracker Green FM, JC-1, and MitoTracker Orange CMTMRos, respectively (Molecular Probes, Life Technologies). CXCR4 antibodies were purchased from BD Biosciences. Phenotyping for mouse transplant chimerism/engraftment analysis was as noted (Mantel et al., 2012; Broxmeyer et al., 2012). Flow cytometric analysis of apoptosis was assessed by activated caspase-3 (Mantel et al., 2007).

Cell Harvests

Hypoxic BM harvest was done in a custom-configured, temperature-, humidity-, O_2-, and CO_2-controlled glove box (Figure S1A; Hypoxic Chamber, Coy) routinely maintained at 3% O_2, 5% CO_2, and N_2 balance. After sacrifice, animals were immediately passed into the chamber through a gassed air lock where femurs were obtained and flushed. All solutions, media, reagents, and plastic ware and pipet tips, and sterile gauze, as well as anything that could come into contact with either the femur or the flushed BM cells, were pre-equilibrated in the hypoxic chamber for at least 18 hr prior to use. Subsequent procedures such as surface marker staining and fixation and colony assay procedures were done inside the chamber (Supplemental Experimental Procedures). CSA harvests (50 μg/ml) were used for BM collection and also injected intraperitoneally at 100 μg into mice 18–24 hr before BM harvest, although subsequent experiments did not demonstrate differences in CSA collection of HSCs whether or not mice were injected with CSA before collection of BM cells in CSA. FK506 and CAT were used at 50 μg/ml. CSA, FK506, and CAT were purchased from Sigma.

Human CB was harvested as reported previously (Broxmeyer et al., 2006), except airtight arterial blood gas-syringes (McKesson Medical-Surgical) equilibrated in the hypoxic chamber were used such that exposure to air was greatly minimized. Syringes were transported from the delivery room to the laboratory in airtight plastic containers and placed back into the hypoxic chamber, often within 10 min of collection. A portion of hypoxic-harvested CB was exposed to air. Centrifugation of hypoxic-harvested BM and CB was done inside the hypoxic chamber or airtight tubes in tabletop centrifuges.

Collection of CB was performed within 5 min of placental delivery. Through a single venipuncture, 15–25 ml of blood was harvested into a 60-cc syringe containing 20 ml of either PBS with heparin (H) (Sigma #H3393) at 1,000 U/ml and CSA (Sigma #P500092) at 50 μg/ml or PBS/H with DMSO (D) at equivalent volume of CSA. Each collection was immediately added respectively to 50 ml of either PBS/H/CSA or DPBS/H/D in a sterile container and mixed thoroughly. Blood was further processed for mononuclear cells and enriched for CD34 cells using standard protocols for Ficoll separation and micro-bead isolation with all solutions respectively containing either CSA at 50 μg/ml or equivalent volume of DMSO.

All studies using human cells were approved by the institutional review board of the Indiana University School of Medicine.

Transplantation

Recipient F1 and NSG mice were continuously fed uniprim feed and irradiated with one dose of 950 or 300 cGy, respectively, 24 hr prior to transplantation with engraftment (Broxmeyer et al., 2012), as detailed in the figure legends. Homing is described in Supplemental Experimental Procedures.

Limiting Dilution Analysis

The frequency of mouse CRUs and human SRCs was analyzed by LDA as previously reported (Antonchuk, et al. 2002; Boitano, et al., 2010). For mouse experiments, increasing doses of C57BL/6 BM cells (CD45.2^{+}) with B6 BoyJ (CD45.1^{+}) competitor cells were transplanted into lethally irradiated (950 cGy) F1 (CD45.2/CD45.1^{+}) recipient mice. For human experiments, increasing doses of CD34^{+} cells were infused into sublethally irradiated (300 cGy) NSG mice. For each LDA experiment, mice were transplanted with either of three cell concentrations, with the second being half of the first and the third being half of the second. Numbers of mice for each dilution are given in the figure legends. HSC frequency was calculated using L-Calc software (STEMCELL Technologies) and plotted using ELDA software (http://bioinf.wehi.edu.au/software/elda/). Poisson statistics was used to calculate the p value for all LDA analyses.

Colony Assays and Tritiated Thymidine Kill Assay

These assays were done as reported elsewhere (Broxmeyer et al., 2012; Mantel et al., 2012). Colonies were scored after incubation at 5% O_2, 5% CO_2 to maximize detectable colony numbers.

Respirometry

Extracellular flux respirometry was done on mouse splenocytes as reported previously (Mantel et al., 2012).

Statistics

Statistical analysis was done using a two-tailed student t test or, where indicated, by ANOVA or a Mann-Whitney test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.04.054.

AUTHOR CONTRIBUTIONS

C.R.M., H.A.O., and H.E.B. co-wrote the paper, provided concepts, and performed experiments and data analysis. B.R.C., X.H., S.C., G.H., E.F.S., M.R.L., and S.M-G. helped with experimental designs and performed experiments. D.M.H., N.F., N.B., R.K., L.M.P., M.I., N. Brustovetsky, N. Bardeesy, J.F., and K.-S.K. provided critical materials, knowledge on their use, and concepts. All authors reviewed the manuscript.

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We would like to dedicate this paper to Dr. Donald Metcalf, a pioneer in the field of hematopoiesis, who recently passed away.

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